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Dear Ms. Andrews

Thank you very much for the opportunity to comment on NTP draft report TR-575. SNF is a major manufacturer of acrylamide monomer both in the United States and worldwide. This document contains preliminary comments and line-by-line review. We are currently conducting a review of the individual animal data (which is incomplete). However, this task is time-consuming and we will not be able to submit the results until after the deadline.

1. Study objective/purpose

NTP states that the object of the study is "to obtain data for meaningful risk assessments". Later, the report states: "Because of data gaps in dose response curves of currently available bioassays, the potential risks to humans that are associates with dietary exposure to acrylamide are difficult to estimate." However, 2 major risk assessments on acrylamide have recently been conducted – the EU Risk Assessment (European Chemicals, 2002) and the EPA Iris Risk Assessment (USEPA 2010) – neither of which recommended conducting another lifetime study on acrylamide. The only real objective of this study is, rather, as a positive control for the glycidamide bioassay and this should be stated up front.

2. Units used for acrylamide exposure

The exposure to acrylamide is expressed as either millimolar (mM) for drinking water or mg/kg diet for feed (except for figure 10). The expression as mM makes sense for the comparison to glycidamide on a molecule to molecule basis. For risk assessment purposes, ppm is a much more useful metric. In the case of the diet, there can be confusion over tables which contain mg/kg as to whether this is animal dose or concentration in diet (and this is not always stated). Use of ppm is a more meaningful unit for risk assessment purposes and is unambiguous. Even in Tables G1-G4 where dose is expressed, a metamorphosis to mg/kg is used.

3. Absence of Pathology Working Group (PWG) reports

Reference is made to pathology analysis and that the final report will contain this report. Nevertheless, the reviewers are asked to review the report without this pathology input. The PWG report should be part of the document reviewed for final approval.

4. Absence of protocol

The protocol for the study was not included with the final report. Frankly we did not notice this until we reviewed the animals body weight data. At the beginning of the chronic study, the range of body weights for 5 week old rats ranged from 52.6–106.5 for males and 60.1–117.2 for females, 11.1–17.8 for male mice and 13.5–21.2 for female mice (page 39). In table 1 (page 42) there are 12 start dates listed for rats and 12 listed for mice. Allocation of animals across these start dates is not described. It is not unlikely that they could have been drawn from the same pool which would explain the large range of body weights. Or males could have been started first followed by females which would explain why females weighed more that males.

5. Mortality

There was substantial mortality in this study with the survival of the high dose being only 13 male and 13 female rats surviving in contrast to 34 controls. It is evident the maximum tolerated dose was well exceeded and this disqualifies the use of study for regulatory/risk assessment purposes. It is all the more surprising that the MTD was exceeded when the reports of 2 lifetime bioassays using the same route of exposure and same strain of rat were available to the NTP.

6. Statistics

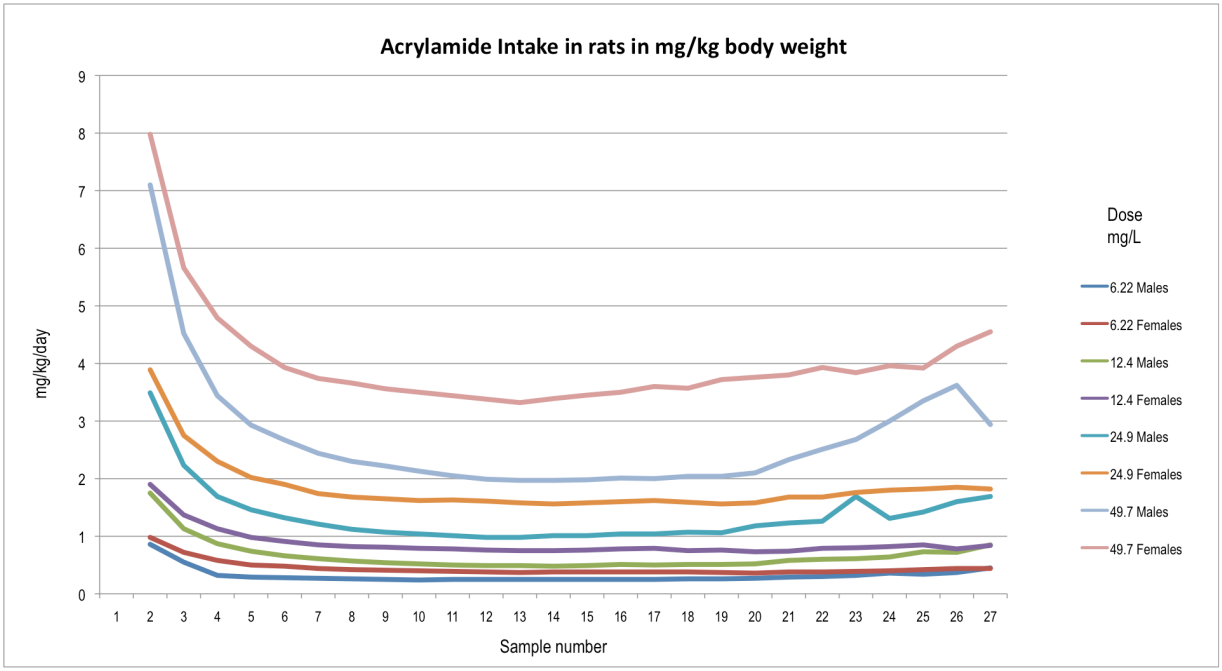
The tables and graphs are generally devoid of statistics. It is difficult to evaluate the significance of the individual findings in this regard.

7. Animal Ethics

This report contains the results of subacute and subchronic rat and mouse feding studies on acrylamide. There are published studies detailing this information without a waste of animals and finances on repeating those studies. This data cannot even be used as a comparison to glycidamide since it is not stable in feed (as stated in the report).

8. Difference in dosing between this study and published studies

There is a fundamental difference between these studies and those which have been published. The published studies were performed on a constant dose of acrylamide throughout the study allowing knowledge of the dose for risk assessment purposes. These studies were conducted on a fixed concentration which represented a variable dose. We are enclosing a graph of the rat dose. Information, such as contained in this graph should be included in the report.



9. Comparison to other alkylating agents

The discussion contains comparison to the toxicology of other alkylating agents, namely *N*-methylolacrylamide, chloroprene, butanediol and ethylcarbamate. In the case of *N*-methylolacrylamide, acrylamide hemoglobin adducts have been detected but there is no literature on the induction of DNA adducts on *N*-methylolacrylamide. *N*-methylolacrylamide is negative in the mouse micronucleus test. In the case of ethylcarbamate, an entirely different set of DNA adducts are induced and these are not relevant to acrylamide toxicology. Furthermore, in each organ where tumors are found, the report points out that there were DNA adducts found in that organ. The report is silent on the presence of DNA adducts in every organ and frequently in greater concentrations than in the target organs.

10. Literature on acrylamide and tunica vaginalis mesotheliomas

The *tunica vaginalis* tumors are highly important to risk assessment, as they are the tumors upon which the EU risk assessment is based. The relevance and interpretation of these tumors are reviewed by Maronpot *et al* (Maronpot, 2009). In this review, they discuss the relevance of time-to-tumor and

incidence on the genotoxic nature of the tumors. They concluded that genotoxic carcinogens which induce *tunica vaginalis* mesotheliomas have incidences >24%. In this study the incidence was 17%. Furthermore, Iatropolis and Williams reported a correlation between size of Leydig cell tumor and the presence of *tunica vaginalis* mesotheliomas (Iatropoulos,1998). Based on this observation, we requested that this correlation be investigated at necropsy by measuring the size of Leydig call tumors and even offered a pathologist to do this. The final report on this study does not even report Leydig cell tumors never mind their size. It is unclear why these tumors are not reported.

11. *Tunica vaginalis* nomenclature

The *tunica vaginalis* mesotheliomas in this study were located in the scrotal sac. They were not tumors of the testes, nor should be referred to as tumors of the testes. We will highlight this in our line by line comments. Similarly, the fibroadenomas observed in the mammary gland are not tumors of the mammary gland and should not be referred to as such. Again we will point this out in our line-by-line comments.

12. *Fibroadenomas of the mammary gland* nomenclature

Similarly, the fibroadenomas observed in the mammary gland are not tumors of the mammary gland and should not be referred to as such. Again we will point this out in our line-by-line comments.

13. *Tunica vaginalis* pathology

Chetty (Chetty, 1992) describes the rat *tunica vaginalis* mesothelioma as a benign papillary tumor. This is consistent with the observations of Iatropolis and Williams (Iatropoulos, 1998) and Damjanov and Friedman (Damjanov, 1998) on tumors induced by acrylamide and discussed earlier. The tumors in this study are described as malignant. Are these morphologically different from those evaluated in the published literature? Since all mesotheliomas have been historically considered malignant, this differentiation of diagnosis is sometimes not considered. As a study conducted for risk assessment, this

is an important distinction as some regulatory bodies do not consider benign tumors in their regulatory scheme.

14. The Tables and graphs are devoid of statistics

It is difficult to interpret the findings, as there are generally no statistics included in the tables. The tables can be improved greatly by including statistics.

15. Murine tumors

The hypothesis upon which this series of studies is based is that acrylamide gets metabolized to glycidamide which in turn alkylates DNA and transforms cells to cancer cells. Mice produce substantially more glycidamide than rats, and therefore should be substantially more sensitive than rats. This did not appear to be the case as the mouse doses were substantially higher than the rat doses and time-to-tumor and incidence did not appear greater in mice than in rats. A discussion of this phase of the study would be highly valuable, particularly as the glycidamide study might yield similar result.

16. Neurotoxicity in Mice

From the report, it appears that there was no neurotoxicity in mice at the high dose. This is from both the cage side observations and the histopathology. This is an important observation and should be emphasized. In rats, neurotoxicity appears to parallel other effects but in the mouse, according to the report, there was no neurotoxicity at the toxic dose.

17. Experimental validation of the mode of action

Maronpot et al (2009), Damjanov and Friedman (1998) and Ship (2006) have reviewed data and slides and concluded that rather than inducing tumors *de novo,* acrylamide accelerates the occurrence of background tumors. In order to test this hypothesis, acrylamide should have been tested on a strain of rat with low background tumor incidence.

Line-By-Line Comments

Page 5, lines 12 and 14 This sentence is repeated – *"Two mice died before the end of the experiment: one male fed 185 mg acrylamide, and one male fed 370 mg acrylamide per kg die*t."

Page 14, Production and Use – Acrylamide in the USA is now manufactured primarily by the enzymatic hydrolysis of acrylonitrile.

Page 24, line 5 From review of the Tareke paper, it appears that the study referred to is a glycidamide study, not acrylamide. This underscores the problem with separating the glycidamide and acrylamide reports. The references refer to glycidamide dosing and not acrylamide.

Page 25, 3rd line from the bottom – The word "pharmacodynamic" is inappropriate as these references deal only with kinetic models and not pharmacodynamic models.

Page 27, last line, 1st paragraph. – This number of alkylated bases should be put in perspective. As there are $3*10^9$ bases per cell and assuming 25% are guanine, this calculates to 0.45-3.75 adducts per cell. Background DNA damage is 19,200 bases per cell per day.

Page 27, 3rd paragraph – With this level of knowledge of the subchronic oral toxicity of acrylamide, how was a subchronic study justified?

Page 28, 1st line – This summary should include data to point out that the reproductive effect, based on crossover studies, is male specific (Chapin and Sloane, 1997; Tyl and Friedman, 2003).

Page 30, line 7 – In the previous line these tumors were described as mesotheliomas of the *tunica vaginalis* and should not be referred to as "testicular tumors".

Page 30, line 14 – There were 100 rats in each group. The 300 should be changed to 100.

Page 30, last paragraph – The best primary reference for epidemiology, Marsh and Schall (1999) should be included. With regard to cancer of the pancreas, the report is correct that it has not been seen elsewhere. In the Marsh study it was singled out by recategorizing the exposures.

Page 32, 2nd paragraph – Von Tungeln stated that *"Only glycidamide increased the frequency of micronucleated reticulocytes and normochromatic erythrocytes. In mice treated on PNDs 1, 8 and 15, the Hprt mutant frequency was increased by 0.70 mmol glycidamide. In mice dosed on PNDs 1-8, 0.70 mmol glycidamide caused extensive mortality; each of the other treatments increased the Tk mutant frequency, whereas acrylamide increased the Hprt mutant frequency."* Acrylamide did not have this effect and this should be removed from the report.

Page 32, last paragraph – It is unclear what data gaps the rat study was designed to fill. While the lack of chronic mouse data on acrylamide is a data gap, there are 2 chronic rats studies which are sufficient for regulatory risk assessment. Outside of being a positive control for the glycidamide study, the NTP has not provided a rationale for conducting the rat study.

Page 36, 2nd line from the bottom – The range of body weights for these studies is enormous and requires explanation. For example, 37.3–103.7 for male rats raises questions concerning the health of the animals.

Page 36, 2nd line from the bottom – As stated above, the range of body weights continues to be excessive. The ranges here are very different from the 2-week. Can this be explained by the staggered start.

Page 39, 1st paragraph – The same comment concerning animal body weight also applies here. However, this is a more complicated issue. There were 12 starting dates for the study listed in Table 1. Since 48 animals and not the usual 50 animals were used, it leads to believe that there was a staggered start to the study. However there is no mention of the staggered start in the report and as said earlier, there is no protocol. This multiple start date was also seen with the other studies but is most striking here.

Page 41, line 4 – The explanation of why it was necessary to convene 'a special neuropathology working group" has been omitted and needs to be included.

Page 41, Table 1 – In this table the various starting dates for each of the studies are listed. Were these staggered starts or were different groups started at each time? Did the animals come from one pool or were there different batches each week. If there were different batches, was batch-to-batch statistics conducted, and if so, where are they?

Page 43, Water – It is stated that Millipore filtered tap water was available. Is this a surrogate for make-up water?

Page 43, Exposure concentrations – We commented earlier about the possibility of misunderstanding use of mg/kg diet and mg/kg body weight. These doses should be labeled "mg/kg diet".

Page 47, line 3 – The Tables are out of order. Table 3 is mentioned before Table 2.

Page 48, line 8 – This should refer to Table 2 and not 3

Page 70 – As we said earlier, a graph of dose vs time would be very informative in this section. That graph should show overlap of doses, which makes interpretation very difficult.

Page 74, 3rd paragraph – The observation of Schwannomas is interesting and because of its unusual occurrence perhaps a photomicrograph might be informative.

Page 74, 4th paragraph – This starts the inaccurate nomenclature of "malignant mesothelioma". As said earlier, these tumors, at least in the acrylamide-treated rats are not malignant as described by Chettty.

Page 75, 3rd paragraph – Combining islet cell adenoma and carcinoma is highly misleading. There was only 1 carcinoma in the study so changing the statistics would be impossible. It is sufficient to say the incidence of islet cell adenomas was significant and no carcinomas were observed.

Page 91, 1st and 2nd paragraphs – The Tables are again out of order. They are cited as 20, 21 and 19. Referring to Table 19 in line three solves this problem.

Page 108, 3rd line – Concluding that adenomas and carcinomas of the Harderian gland are increased is misleading. There were only 2 carcinomas in the study and these had virtually no impact on the statistics. The impact of this sentence on the regulatory interpretation of this study is substantial.

Page 117, 3rd line – This is another example of poor nomenclature about *tunica vaginalis* mesotheliomas. Rather than testes, this should read "testicular *tunica vaginalis* mesotheliomas", heart should read "schwannomas in the heart". In the following line, it should read "mammary gland fibroadenomas"

Page 118, 2nd paragraph – Justification of a response based on DNA adducts in the target tissue needs some qualification. All tissues contain DNA adducts to more or less the same extent.

Page 119, 2nd paragraph – This report does not in any way lend support to the concept that acrylamide is carcinogenic through a similar pathway [as glycidamide].

Page 119, last paragraph – This is misleading. There have not been adduct analyses from the *tunica vaginalis* where the tumors are found but rather a gemisch of testicular cells. A better reference would have been Marlowe et al (1986) where they showed that the acrylamide was bound to the germinal tissue.

Page 120, 2nd paragraph, last line. – This is a highly inaccurate statement and needs to be either removed or corrected. Recategorizing the Marsh data changes the statistics. Marsh conducted a follow-up of this study and no increase was observed.

Page 112, 2nd paragraph. There are more than 4 mouse Harderian gland carcinogens, including the following:

Acrylonitrile; Benzene; Benzidine.2HCl; 2,2-Bis(bromomethyl)-1,3-propanediol, technical grade; 1,3-Butadiene; Captafol; 3-Chloro-2-methylpropene; Chloroprene (>96% chloroprene); Cupferron; Dichloroacetylene; Ethylene oxide; Gentian violet; Glycidol; Iodinated glycerol; Isoprene; N-Methylolacrylamide; Nitromethane; 4,4′-Oxydianiline; Tetrachloroethylene; 1,2,3-Trichloropropane; 2,4,6 Trinitro-1,3-dimethyl-5-tert-butylbenzene; Vinyl fluoride

Does this list show that acrylamide acts through the glycidamide pathway? In the glycidamide report, this suggestion can be made but absent that data here it is premature to speculate. Of these, benzene, 2,2-Bis(bromomethyl)-1,3-propanediol, technical grade; 1,3-Butadiene; Chloroprene (>96% chloroprene); Ethylene oxide; Glycidol; *N*-Methylolacrylamide; and Nitromethane also cause lung tumors in mice.

Literature Cited

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